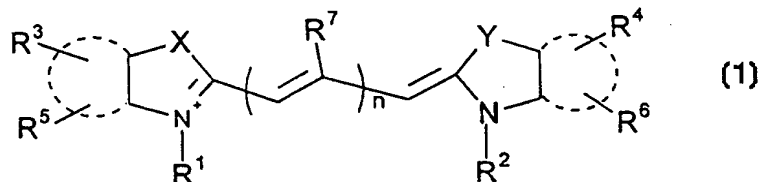




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(54) Title: CYANINE DYES



(57) Abstract

A cyanine dye having structure (1) has at least three positively charged N or P or S atoms, and also preferably has a reactive or functional group by which it may be linked to a biomolecule or a solid surface.

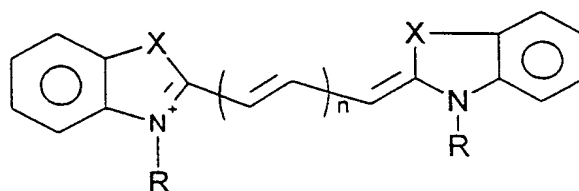
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CYANINE DYES

5 The cyanine dye class has proved to be an extremely bright and versatile class of dyes in both photographic and biological applications. The addition of sulphonic acid and attachment of functionality for conjugation have allowed them to be fully exploited for biological research applications. The addition of sulphonic acids for additional water
10 solubility and enhanced brightness has led to the dyes becoming overall neutral or negatively charged. As described in US Patents 5,268,486 and 5,486,616, the basic cyanine structure has a +1 overall positive charge e.g.

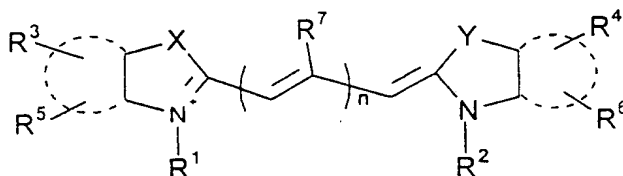


overall +1 charge

15

In certain applications dyes having several positively charged atoms can be of benefit. This invention addresses that need.

The invention provides a cyanine dye having the structure



(1)

20

- 2 -

where the dotted lines represent the carbon atoms necessary for a one ring or a two or three fused ring system with 5 or 6 carbon atoms in each ring and R^3 , R^4 , R^5 and R^6 attached to the rings,

X and Y are independently selected from O, S and CR_2^8 ,

5 where R^8 is $C_1 - C_4$ alkyl,

n is 1, 2 or 3,

at least one of R^1 , R^2 , R^3 , R^4 , R^5 , R^6 and R^7 optionally comprises a reactive or a functional group,

at least one of R^1 , R^2 , R^3 , R^4 , R^5 , R^6 and R^7 incorporates one
10 to five positively charged nitrogen or phosphorus or sulphur atoms,

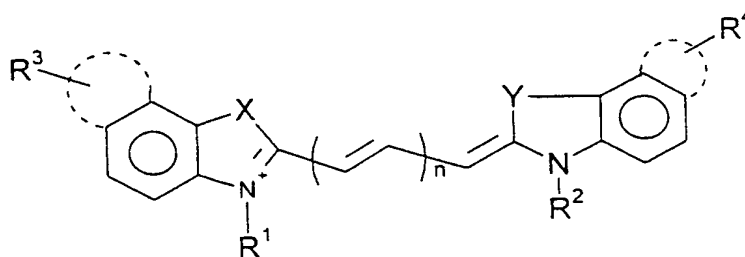
any remaining R^3 , R^4 , R^5 and R^6 is independently selected from H, SO_3^- , Cl, Br, OR^9 and SR^9 , where R^9 is $C_1 - C_{10}$ alkyl or aryl or aralkyl,

any remaining R^1 and R^2 is independently selected from
15 $C_1 - C_{10}$ alkyl or aryl or aralkyl either unsubstituted or substituted by SO_3^- ,

any remaining R^7 is selected from H and $C_1 - C_{10}$ alkyl or aryl or aralkyl either unsubstituted or substituted by SO_3^- ,

provided that at least two positively charged atoms selected from nitrogen and phosphorus and sulphur are present in the groups R^1 ,
20 R^2 , R^3 , R^4 , R^5 , R^6 and R^7 .

Preferably the cyanine dye has the structure (2)



(2)

- 3 -

Preferably the cyanine dyes have an overall positive charge of +2 to +6. The overall charge of the dye may be considered as the number of positively charged nitrogen (or phosphorus or sulphur) atoms minus the number of sulphonate (or carboxyl or phosphate) groups. Thus
5 for example, a dye having 3 positively charged nitrogen atoms and 0 or 2 or 4 sulphonate groups would have an overall charge of +3 or +1 or -1, respectively. The extent to which an atom or group is charged may depend on the pH of its environment.

Preferably a reactive or functional group is present as a
10 structure -L-Q where L is a linker and Q is the reactive or a functional group. A reactive group of the dye can react with a functional group of a target molecule; or a functional group of the dye can react with a reactive group of a target molecule; whereby the target molecule becomes labelled by the dye. Preferably Q is a functional group selected from primary
15 amine, secondary amine, hydrazine derivatives, hydroxylamine derivatives, and pyrazolone. Alternatively a functional group may be selected from sulphhydryl, carboxyl, hydroxyl, thiophosphate, imidazole and carbonyl including aldehyde and ketone.

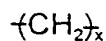
Preferably a reactive group is selected from succinimidyl
20 ester, isothiocyanate, dichlorotriazine, isocyanate, haloacetamide, maleimide, sulphonyl halide, acid halide, alkylimido ester, arylimido ester, carbodiimide, phosphoramidite, anhydride and acyl azide.

By virtue of these functional and reactive groups, the cyanine dyes of the present invention are combined with target materials to form
25 conjugates. Suitable target materials may include antibodies, antigens, proteins, carbohydrates, lipids, nucleotides, nucleic acids, polymer particles or glass beads. Thus for example, cyanine dyes having the preferred functional groups mentioned above are suitable for reacting with carbohydrates to form conjugates therewith.

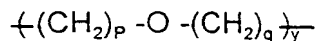
30 L is a linker, which may contain 1-60 chain atoms selected

- 4 -

from C, N, O, S and P, e.g. a straight chain of 1-30 carbon atoms in which are incorporated one or more N, O, S or P atoms. For example the linker may be



5



where x is 1-30, preferably 1-10,

p is 1-5,

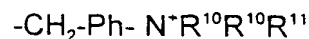
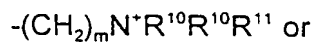
10

q is 0-5 and

y is 1-5.

Present in the cyanine dye of the invention is a branched or straight chain incorporating 1-5 positively charged nitrogen or phosphorus or sulphur atoms. (Some or all of these positively charged N or P or S atoms may be present in the linker group L.). Preferably each positively charged atom is a nitrogen atom is provided by a quaternary ammonium group, or alternatively by a protonated tertiary amino group, a guanidinium group, an imidazole group or a pyridinium group. Positively charged P and S atoms may be provided by phosphonium ions and sulphonium ions respectively. Preferably a branched or straight chain incorporating one to five positively charged nitrogen atoms is up to 60 chain carbon atoms and has the structure

25



where m is 1 to 4,

R¹⁰ is C₁ - C₁₀ alkyl,

and R¹¹ is C₁ - C₁₀ alkyl or -(CH₂)_mN⁺R¹⁰R¹⁰R¹¹.

Or the linker group L and/or the chain incorporating positively charged nitrogen atoms may comprise one or more natural or artificial

30

- 5 -

amino acid residues. It is a simple matter to introduce any number e.g.
 1 - 20 of lysine residues, and if desired to quaternise the ϵ -amino groups.
 Such linkers may contain the grouping $-(CO.NHW)_r-$ where r is preferably
 1 to 6 and W is aminoalkyl or quaternised aminoalkyl such as $-(CH_2)_4NH_2$
 5 or $-(CH_2)_4N^+R^{10}_3$ where R^{10} is $C_1 - C_{10}$ alkyl.

At least two positively charged nitrogen or phosphorus or
 sulphur atoms and preferably at least one reactive or functional group are
 present in pendant groups attached to the core structure of the dye. They
 may be positioned on the same group or different groups $R^1, R^2, R^3, R^4, R^5,$
 10 R^6 and R^7 .

Preferably the cyanine dye has the structure (2)

wherein X and Y are $C(CH_3)_2$,

n is 1 or 2,

R^1 is $-(CH_2)_5-COOH$,

15 R^2 is $-(CH_2)_3-N^+(C_2H_5)_3$ or
 $-(CH_2)_3-N^+(CH_3)_2-(CH_2)_3-N^+(CH_3)_2(C_2H_5)$,
 and R^3 and R^4 are H .

The dyes described in the experimental section below have
 quaternary ammonium ions attached for the specific purpose of increasing
 20 the overall positive charge of the dye. The dyes have been made as
 carboxylic acids to enable their use in labelling DNA or other biological
 molecules via active ester derivatives. The increased positive charge may
 be beneficial in electrostatic interactions with DNA in certain specific
 applications and in providing labelled nucleotides having particular charges
 25 for other purposes. It is also envisaged that at least one sulphonic acid
 group can be added to any of the +3 (or more) dyes to give a dye that may
 have an overall positive or negative charge or may be neutral and may
 have improved photostability and brightness. This improvement is useful in
 applications such as difference gel electrophoresis technology as
 30 described in WO 96/33406 where the overall charge on the dye is of

- 6 -

importance.

The carboxylic acid derivative can be reacted: either with diamine species such as 1,3-diaminopropane or ethylene diamine to provide a primary amine functional group; or with a protected hydrazine to generate a corresponding hydrazide which can be deprotected; e.g. for linking to carbohydrates. The addition of extra quaternary amino groups and the controlled use of sulphonic acid groups can lead to a range of dyes having overall positive charges ranging up to 6 or even more.

Cy3 ($n=1$) and Cy5 ($n=2$) and Cy7 ($n=3$) dyes have the added advantage of allowing multiplexing i.e. the use of mixtures of targets labelled with different dyes for simultaneous analysis. This concept can also be increased by varying the intermediate derivative between indole, thiazole and oxazole derivatives, and by altering the number of fused aromatic rings to the dye.

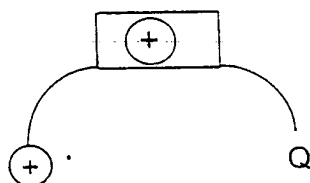
Chemical Strategy

This section shows the chemistry envisaged in making cyanine dyes having positive charges from 2 to 6. Each numbered paragraph starts with a general picture of a cyanine dye shown as a rectangle, having a single positive charge shown as + within a circle. To two corners of the rectangle are attached curved lines which may comprise at least one positive charge and/or at least one functional or reactive group Q or Q'; these curved lines correspond to R^1 , R^2 , R^3 , R^4 , R^5 , R^6 and R^7 , most usually R^1 and R^2 , in the structures (1) and (2) shown above and in the claims. Some of the cyanine dyes have been made and are described below in the Examples; others are in preparation or are envisaged.

- 7 -

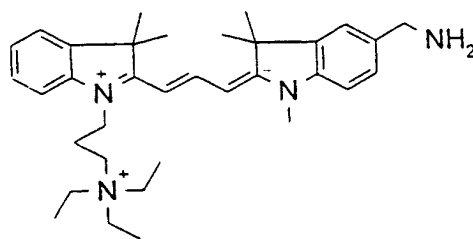
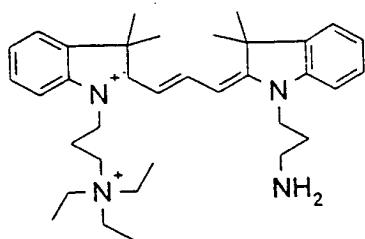
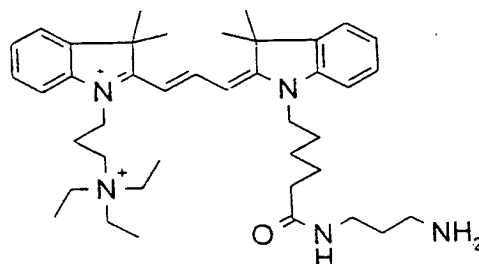
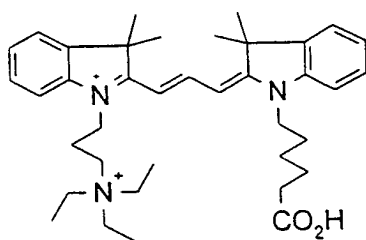
+2 DYES

1.

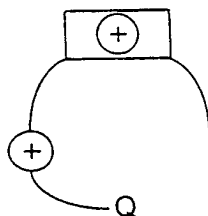


- The dye carries an inherent +1 charge. A second + charge is located on a chain attached to one of the dye N atoms. A functional or reactive group Q terminates a chain attached to the other dye N atom.

Examples:



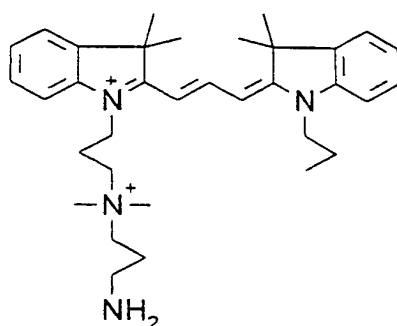
2.



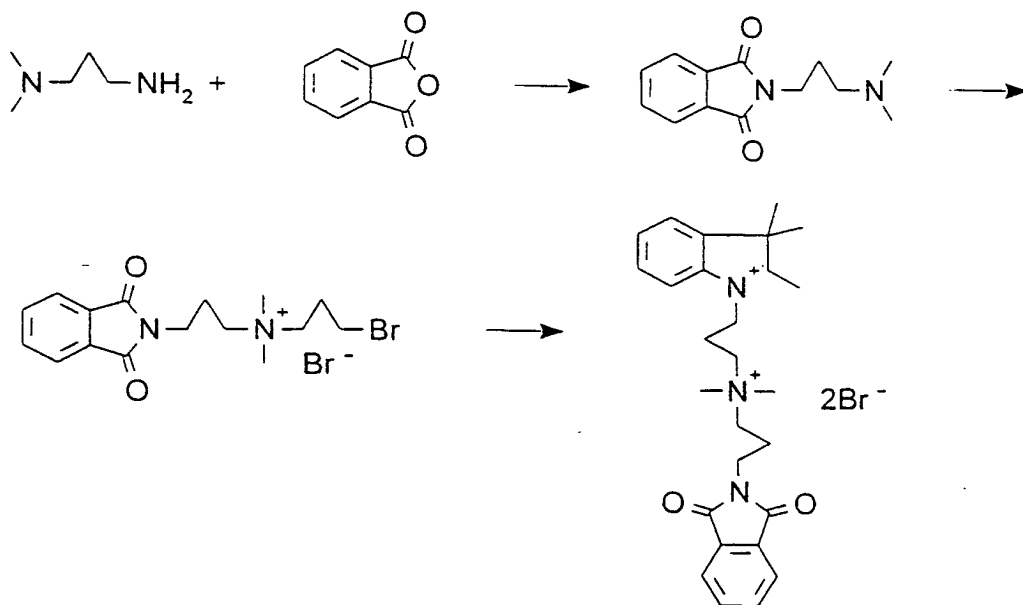
- 8 -

The dye carries an inherent +1 charge. A second + charge is located on a chain attached to one of the dye N atoms. A functional or reactive group Q terminates the same chain.

5 Example:



Synthesis of +2 intermediate:

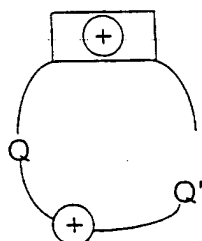


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This intermediate is used to make the protected dye. The phthalimide is removed by hydrolysis in hydrochloric acid to give the amine dye.

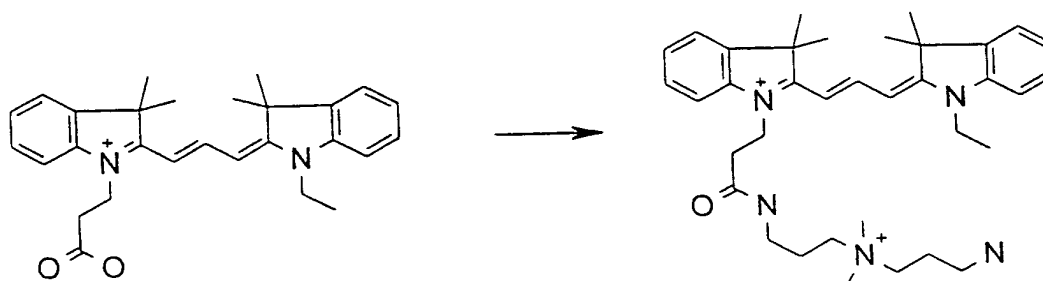
- 9 -

3.



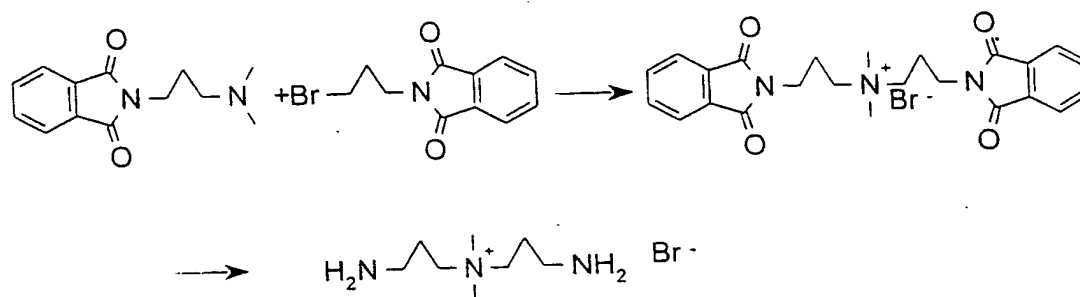
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A +1 monoreactive dye is extended with a linker, which itself contains the second + charge. A possible example is as follows:



Projected synthesis of +1 linker:

10

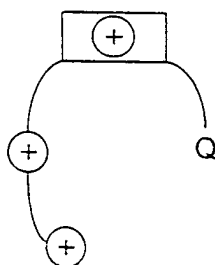


- 10 -

+3 DYES

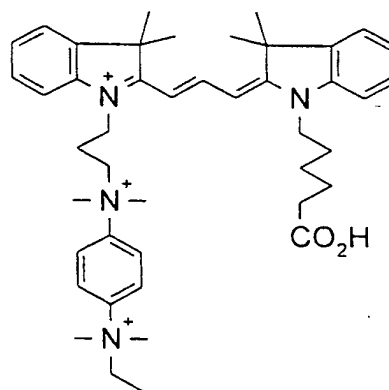
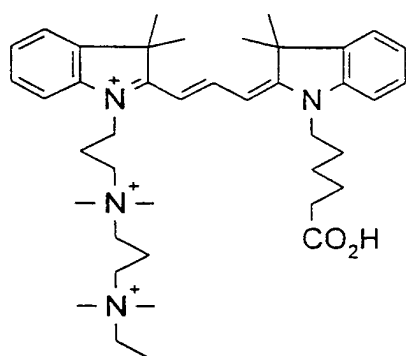
These examples are analogous to those for +2 dyes.

5 1.



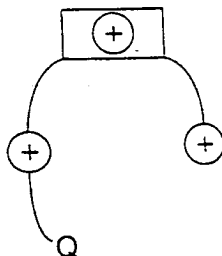
The dye carries an inherent +1 charge. The two extra + charges are located on a chain attached to one of the dye N atoms. A functional or reactive group Q terminates a chain attached to the other dye N atom.

Examples:



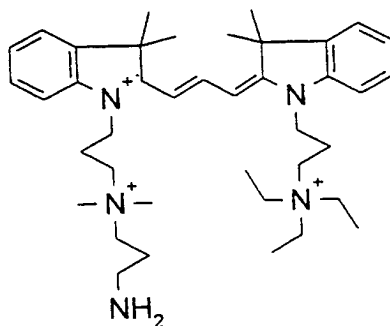
- 11 -

2.



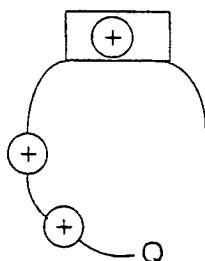
The dye carries an inherent +1 charge. There is one extra + charge on each chain attached to the dye N atoms. A functional or reactive group Q terminates one of these chains.

Example:



- 12 -

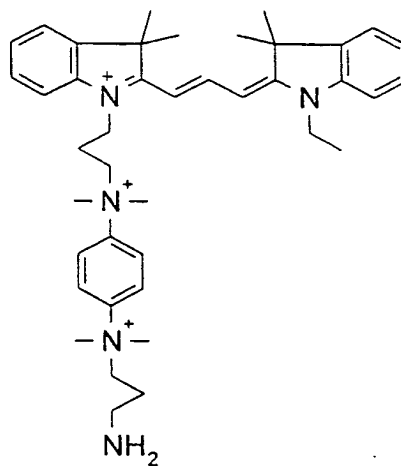
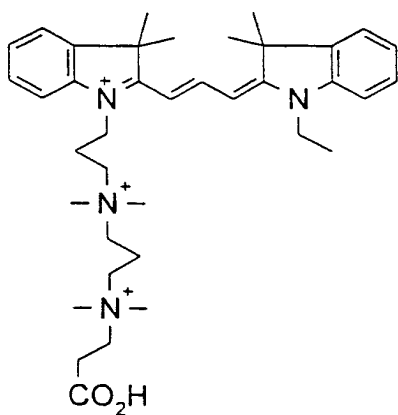
3.



The dye carries an inherent + charge. The other two + charges are both on one chain off a dye N atom; this chain also includes a functional or reactive group Q. This requires a +3 charged intermediate containing a functional or reactive group.

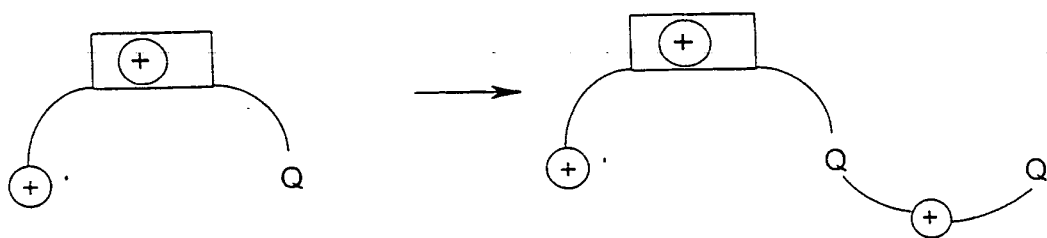
Examples:

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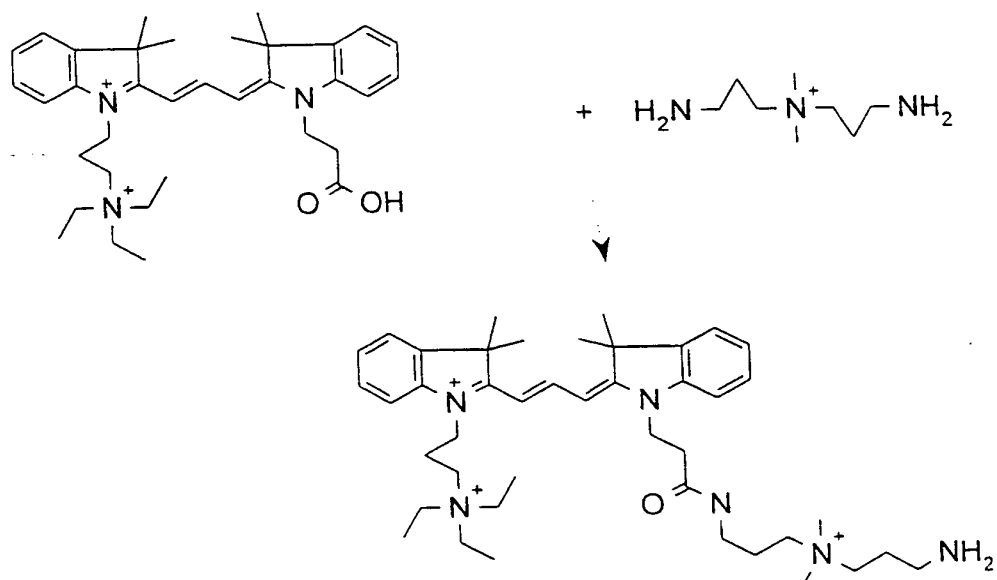
- 13 -

4. Conversion of a +2 dye to a +3 dye by addition of a +1 linker:

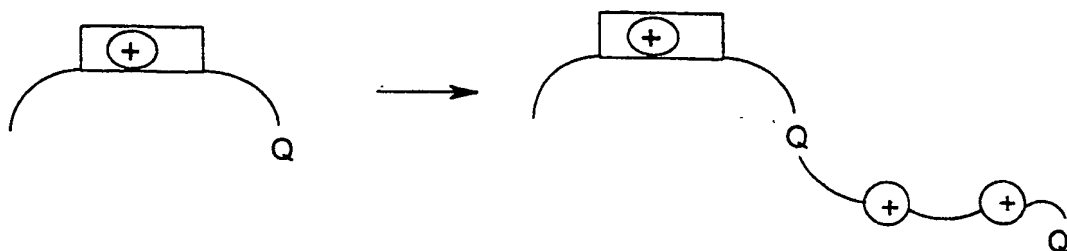


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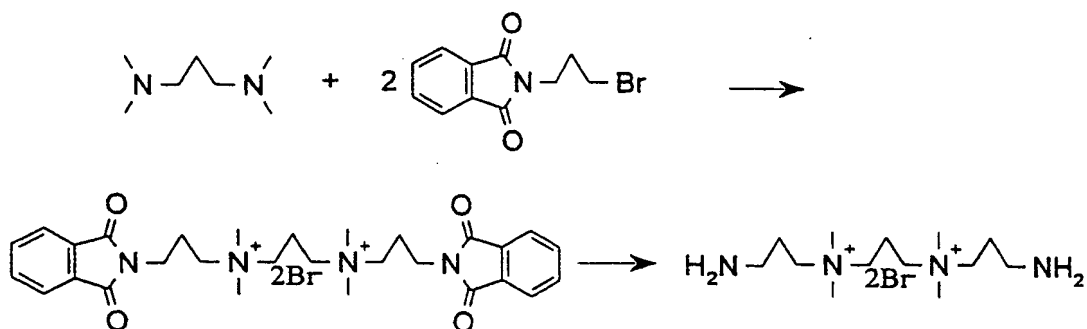
Example:



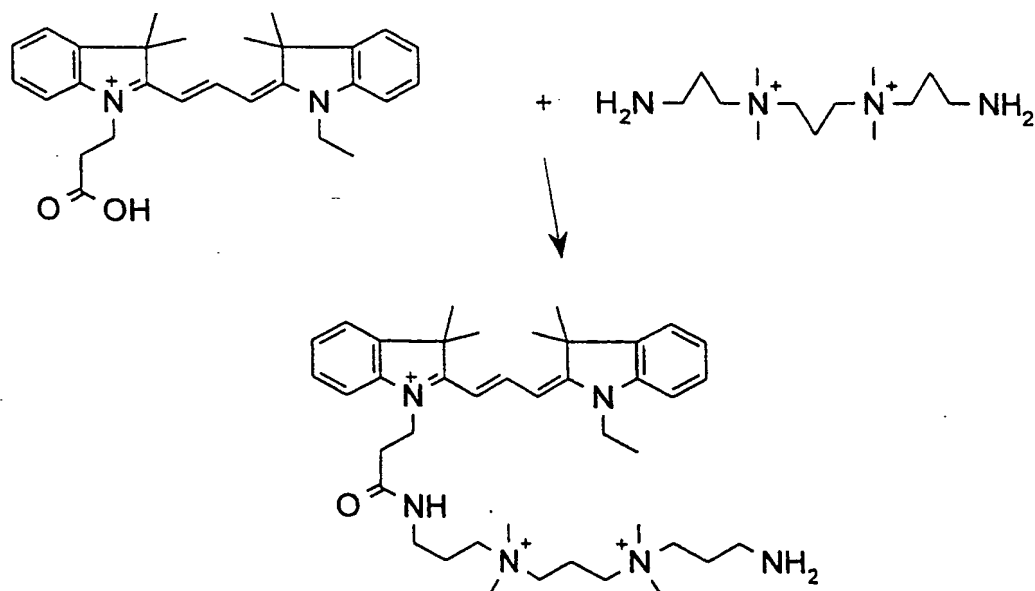
5. Conversion of a +1 dye to a +3 dye by addition of a +2 linker.



Examples:

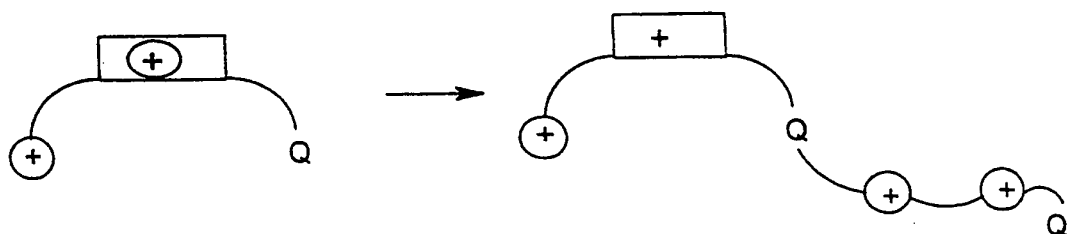


5 +3 dye:

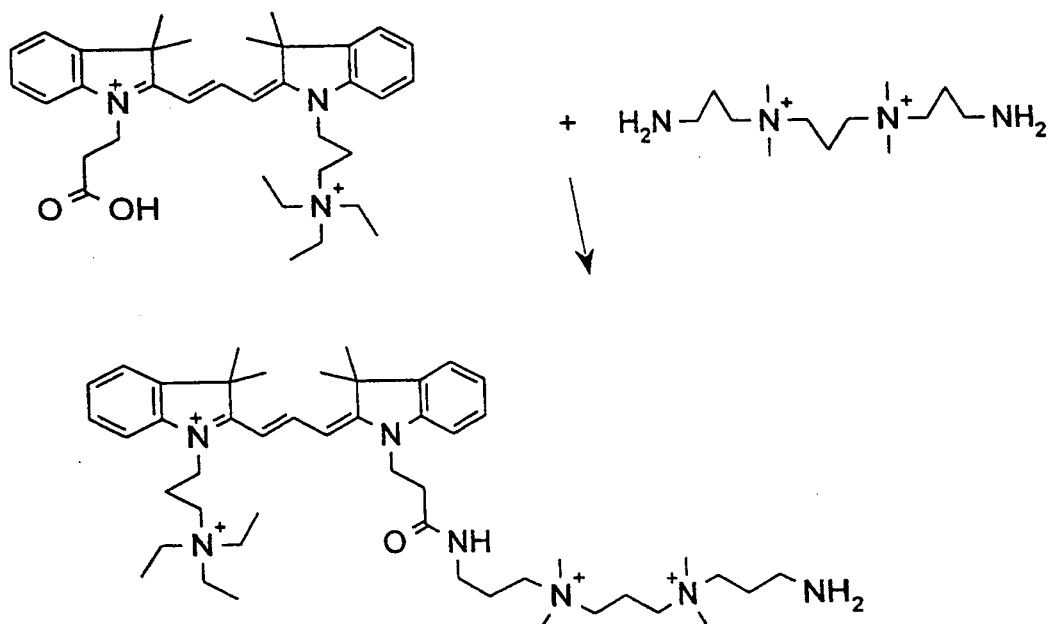


+4 DYES

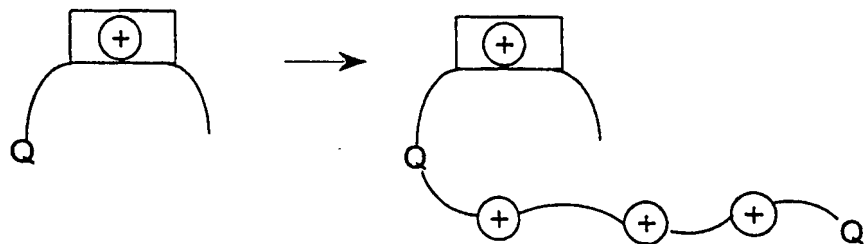
1. Conversion of a +2 dye to a +4 dye by addition of a +2 linker:



5 Example:

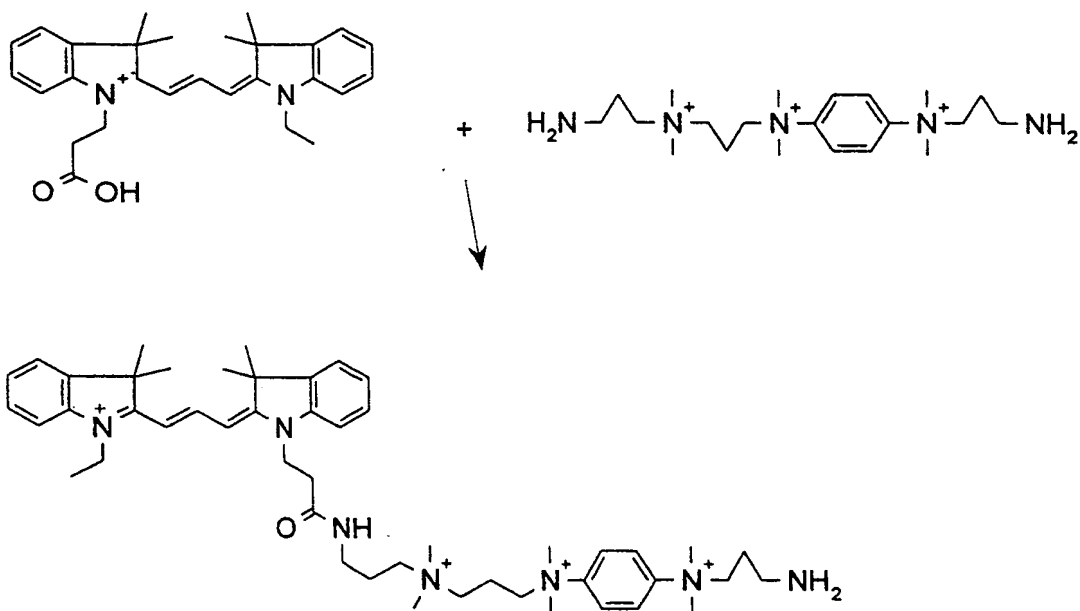


2. Conversion of a +1 dye to a +4 dye by addition of a +3 linker:



5

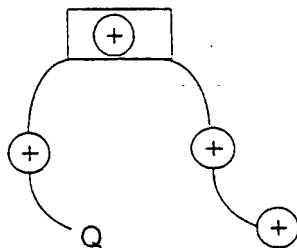
Example:



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- 17 -

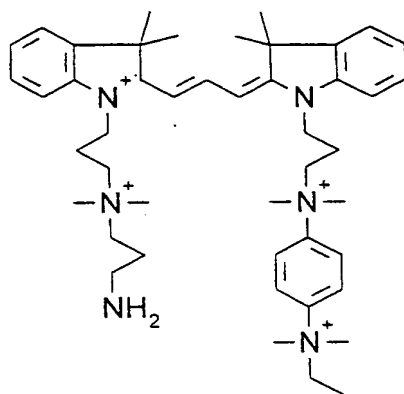
3.



Requires a +2 intermediate with a reactive group and a +3 intermediate.

5

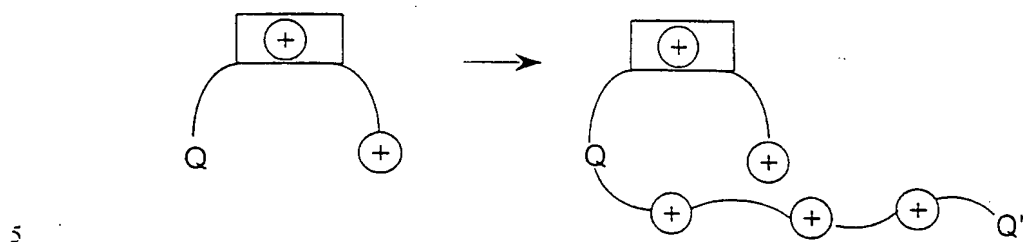
Example:



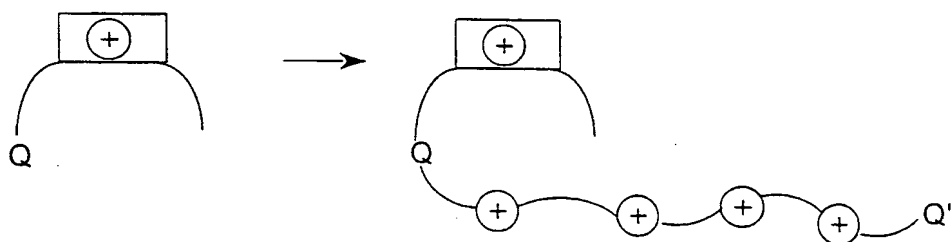
- 18 -

+5 and +6 DYES

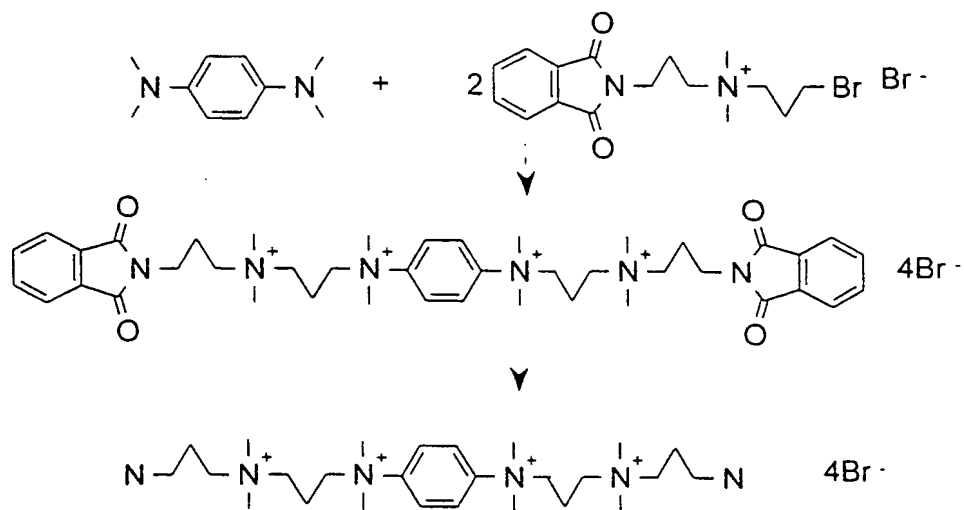
1. Conversion of a +2 dye to a +5 dye by addition of a +3 linker:



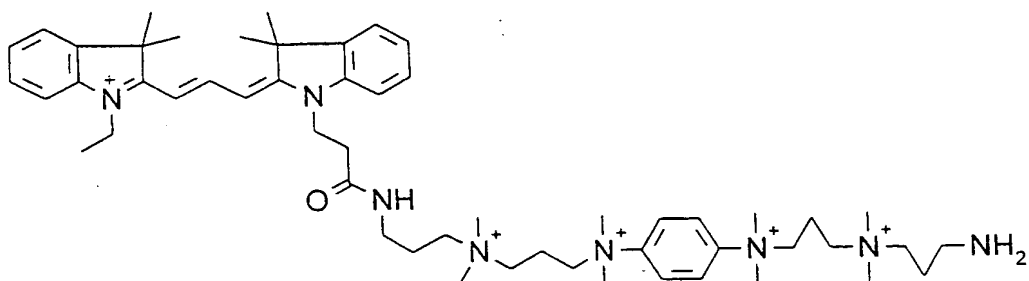
2. Conversion of a +1 dye to a +5 dye by addition of a +4 linker:



10 The +4 linker:

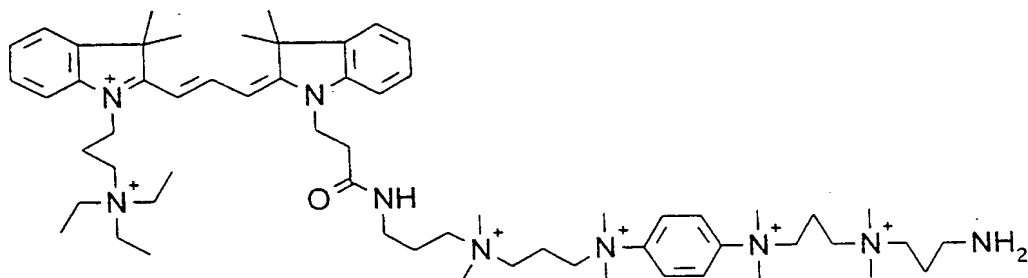
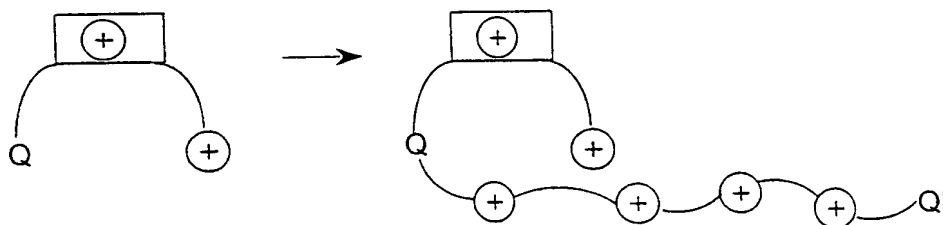


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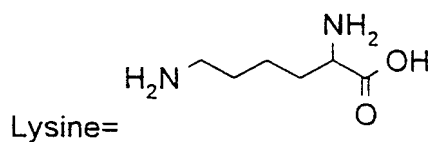
3. Conversion of a +2 dye to a +6 dye by addition of a +4 linker:

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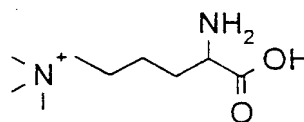


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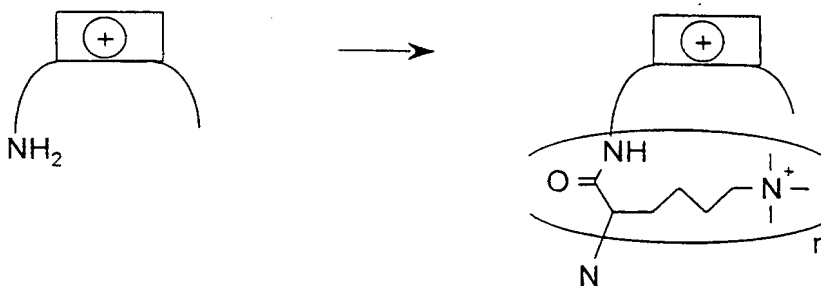
- 20 -

LINKER CHAINS BASED ON POLY-LYSINE

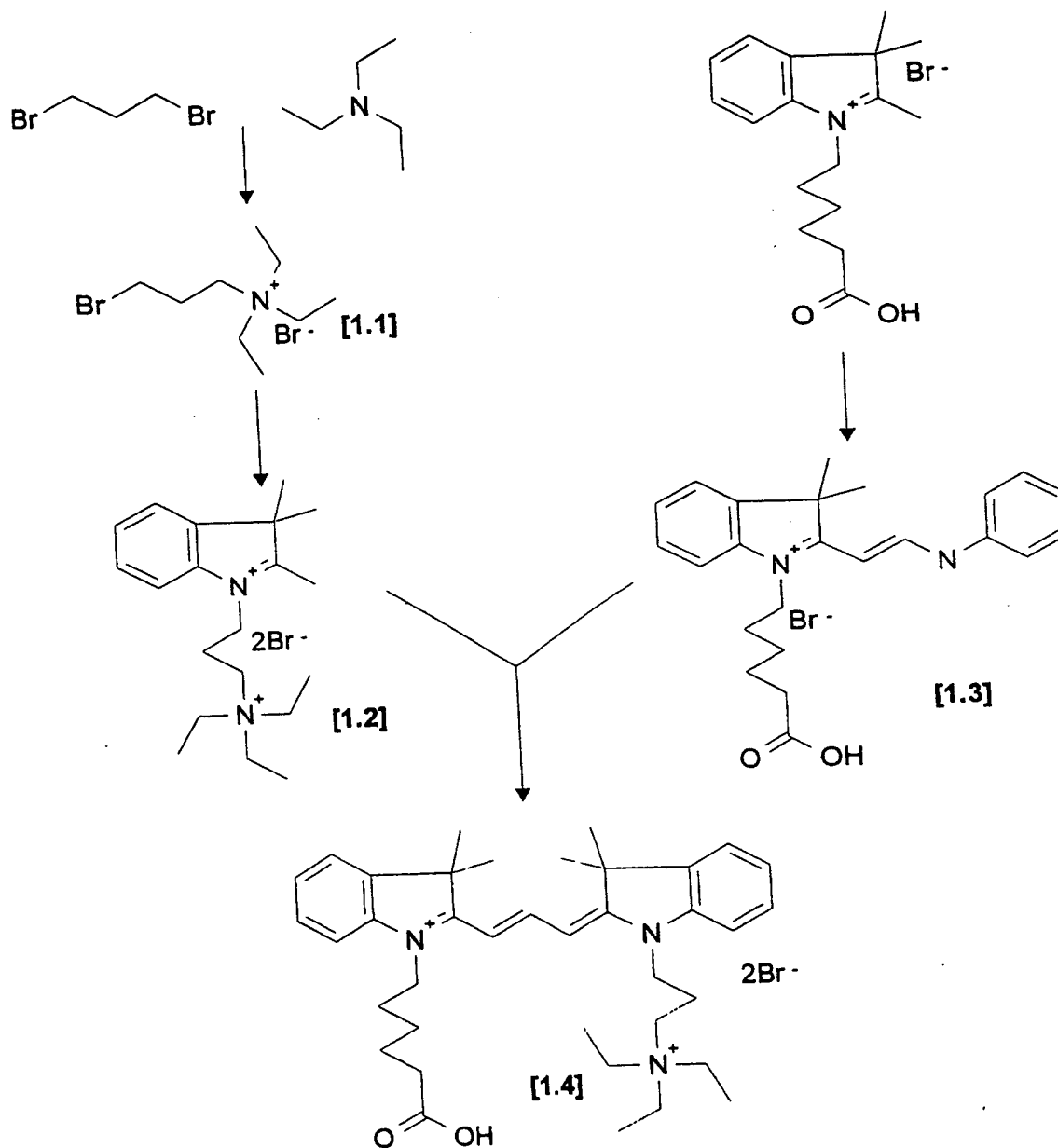
. Also available:



- 5 Construct oligomers on a solid support and couple to +1 dyes to give n+ dyes:



(n+1) charged dye
where n is 1-5 or 6

Example 1**+3 Charged dye, including +1 charged diamine linker (BOC-protected)****5 i) Preparation of a +2 charged carboxy Cy3 dye****N-(3-Bromopropyl)triethylammonium bromide [1.1]**

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1,3-Dibromopropane (20.0g, 100mmol) and triethylamine (5.06g, 50mmol) were mixed in dry toluene (50ml). This solution was heated at 100°C under nitrogen atmosphere for 4hrs, during which time a thick white solid precipitated. The mixture was then cooled and the solid collected by filtration, washed with toluene and ether and dried under vacuum at 50°C to give the title compound **[1.1]**, 5.0g (36%).

δ_H (300MHz, DMSO) broad peaks. 1.17 (9H, $3 \times N^+-CH_2-CH_3$), 2.15 (2H, $BrCH_2CH_2CH_2-$), 3.26 (8H, $4 \times N^+-CH_2$), 3.62 (2H, $Br-CH_2-$).

10 **1-((3-Triethylammonium)propyl)-2,3,3-trimethylindolium dibromide [1.2]**

Freshly distilled 2,3,3-trimethylindolenine (0.8g, 5mmol) and N-(3-bromopropyl)triethylammonium bromide **[1.1]** (1.52g, 5mmol) were mixed and placed under an argon atmosphere. The mixture was then heated at 140°C for 1.5hrs, giving a deep red viscous melt, which solidified to a glass on cooling. It was ground to a powder under diethyl ether; this was collected by filtration, triturated with boiling acetone and recrystallised from methanol / acetonitrile to give the title compound **[1.2]** as a pale pink powder, 795mg (34%).

20 δ_H (300MHz, DMSO) 1.22 (9H, t, J 6.6Hz, $3 \times N^+-CH_2-CH_3$), 1.55 (6H, s, indole C3Me₂), 2.21 (2H, m, $-CH_2CH_2CH_2-$), 2.92 (3H, s, indole C2-Me), 3.27 (6H, q, J 6.6Hz, $3 \times N^+-CH_2-CH_3$), 3.51 (2H, ~t, $-CH_2-NEt_3$), 4.57 (2H, ~t, indole N^+-CH_2-), 7.64 (2H, m), 7.86 (1H, d, J 6.5Hz), 8.12 (1H, d, J 7.3Hz).

25 **1-(5-Carboxypentyl)-2-(N-phenyl-2-aminovinyl)-3,3-dimethylindolium bromide [1.3]**

1-(5-carboxypentyl)-2,3,3-trimethylindolium bromide (1.77g, 5mmol) and N,N'-diphenylformamidine (1.96g, 10mmol) were mixed in acetic acid (15ml); the resulting mixture was then heated at reflux. The

- 23 -

reaction was monitored by UV/VIS spectroscopy (methanol solution, product absorbance λ_{\max} 398nm) and TLC (silica. Methanol, 20 : dichloromethane, 80; product runs as a yellow streak, R_f 0.1-0.25). After 2.5hrs the orange-red solution was then left to cool over 16hrs, then the solvent was removed under reduced pressure. The residue was purified by flash chromatography (silica. 5-20% methanol / dichloromethane) to give the title compound [1.3] as a yellow-orange foam after drying, 1.5g (66%).

UV/VIS λ_{\max} (MeOH) 398nm

δ_H (300MHz, $CDCl_3$) 1.53 (2H, m), 1.63 (6H, s, indole C3 Me₂), 1.70 (2H, m), 1.78 (2H, m), 2.35 (2H, t, J 7.0Hz, $-CH_2-CO_2H$), 3.99 (2H, t, J 7.4Hz, indole N⁺-CH₂-), 6.3 (2H, broad, NH + CO₂H), 7.0-7.45 (10H, m), 8.40 (2H, d, J 12.5).

1-(Carboxypentyl)-1'-((triethylammonium)propyl)-indocarbocyanine dibromide [1.4]

1-(5-Carboxypentyl)-2-(N-phenyl-2-aminovinyl)-3,3-dimethylindolium bromide [1.3] (229mg, 0.5mmol) was dissolved in anhydrous pyridine (5ml) to give an orange solution. To this was added acetic anhydride (0.5ml) and the mixture stirred for 5mins. 1-((3-Triethylammonium)propyl)-2,3,3-trimethylindolium dibromide [1.2] (231mg, 0.5mmol) was then added and the mixture warmed briefly to aid dissolution of the solid. A deep red-pink colour soon formed.

After 2hrs stirring the solvent was removed under reduced pressure and the residue dried under high vacuum. It was then purified by flash chromatography (grade I neutral alumina. 5-20% methanol / chloroform), isolating the major pink component, to give the title dye [1.4] as a red solid, 278mg.

TLC (C-18 silica. Acetic acid, 50: water, 45: methanol, 5: R_f pink spot 0.55).

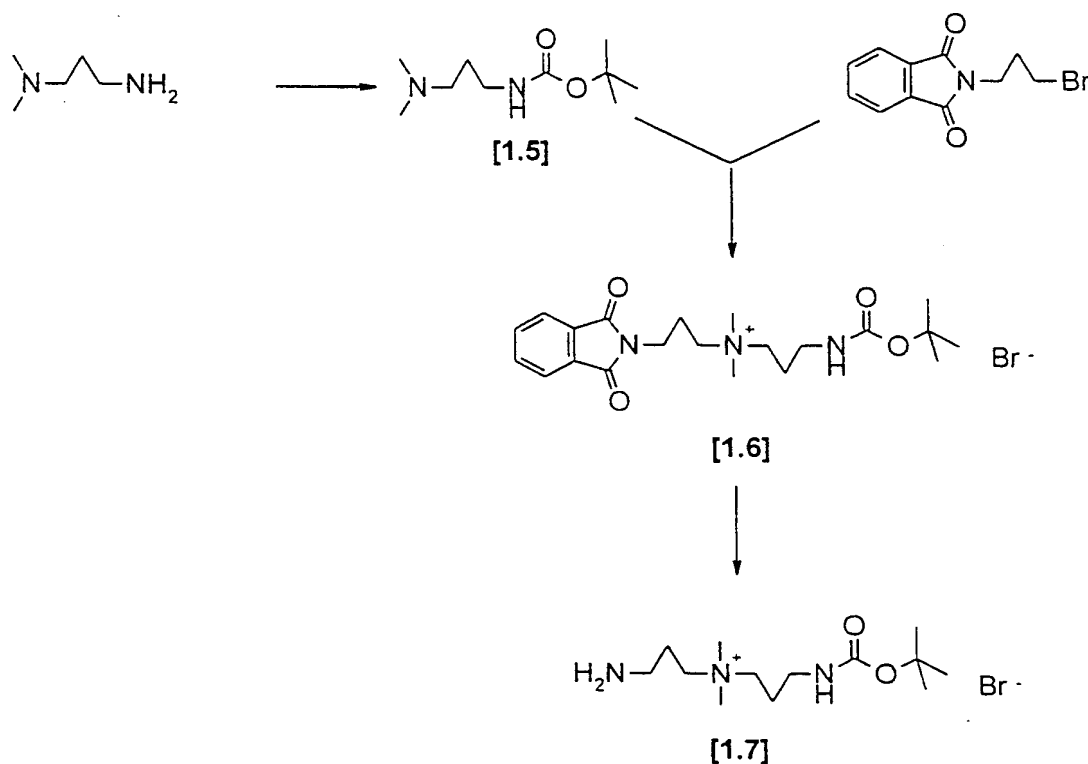
UV/VIS λ_{\max} (MeOH) 548nm.

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Fluorescence (MeOH) λ_{ex} 548nm; λ_{em} 564nm.

δ_{H} 300MHz, CD_3OD) 1.17 (9H, t, J 6.5Hz, $3 \times \text{N}^+-\text{CH}_2\text{CH}_3$),
 1.32 (2H, m), 1.72-1.88 (16H, m), 2.2 (2H, t, J 7.3Hz, $-\text{CH}_2\text{CO}_2\text{H} + 2\text{H}$,
 broad, $\text{N}^+-\text{CH}_2\text{CH}_2\text{CH}_2\text{NEt}_3$), 3.37 (6H, q, J 6.5, $3 \times \text{N}^+-\text{CH}_2\text{CH}_3$), 3.50 (2H,
 5 m, $-\text{CH}_2\text{NEt}_3$), 4.19+4.26 (2H, t, J 7.7Hz, + 2H, t, J 7.3Hz, $2 \times$ indole
 N^+-CH_2-), 4.59 (1H, broad, $-\text{CO}_2\text{H}$), 6.55 (1H, d, J 13.6Hz, methine $=\text{CH}-$),
 6.61 (1H, d, J 13.2Hz, $2 \times$ methine $=\text{CH}-$ indole), 7.28-7.49 (6H, m), 7.54-58
 (2H, m), 8.57 (1H, ~t, $J \sim 13.4\text{Hz}$, $=\text{CH}-\text{CH}=\text{CH}-$).

10 ii) Preparation of a +1 charged diamine linker (BOC-protected)



N-(t-Butoxycarbonyl)-N-(3-dimethylamino)propylamine [1.5]

3-Dimethylamino-1-propylamine (2.04g, 20mmol) was mixed
 15 with dichloromethane (5ml); the resulting solution was cooled to 0°C using
 an ice-water bath. To this was added a solution of di-*t*-butyl dicarbonate

- 25 -

(4.4g, 20mmol) in dichloromethane (15ml); the mixture was then allowed to warm to room temperature. After 2hrs the solution was washed twice with water, then dried (MgSO_4), filtered and the solvent evaporated under reduced pressure to give a colourless oil. Drying under high vacuum gave the title compound, 3.07g (76%).

δ_{H} (300MHz, CDCl_3) 1.44 (9H, s), 1.64 (2H, quin, J 6.8), 2.21 (6H, s), 2.31 (2H, t, J 7.0), 3.17 (2H, broad quartet) and 5.15 (1H, broad s).

N-(phthalimidopropyl)-N-((t-butoxycarbonylamino)propyl)-N,N-dimethylammonium bromide, [1.6]

N-(t-Butoxycarbonyl)-N-(3-dimethylamino)propylamine [1.5] (3.03g, 15mmol) and 3-(bromopropyl)phthalimide (4.02g, 15mmol) were dissolved in dry toluene (8ml). The resulting solution was heated at 50°C for 16hrs, during which time a glassy resin formed on the inside of the flask. The mixture was cooled and the liquors decanted; the residue was triturated with ether to give a glassy powder. Dried under high vacuum to give the title compound [1.6], 4.12g (58%).

δ_{H} (300MHz, CD_3OD) 1.42 (9H, s), 1.91 (2H, m), 2.18 (2H, m), 3.07 (6H, s), 3.12 (2H, t, J 6.6), 3.3-3.5 (4H, m), 3.80 (2H, t, J 6.4) and 7.80-7.90 (4H, m).

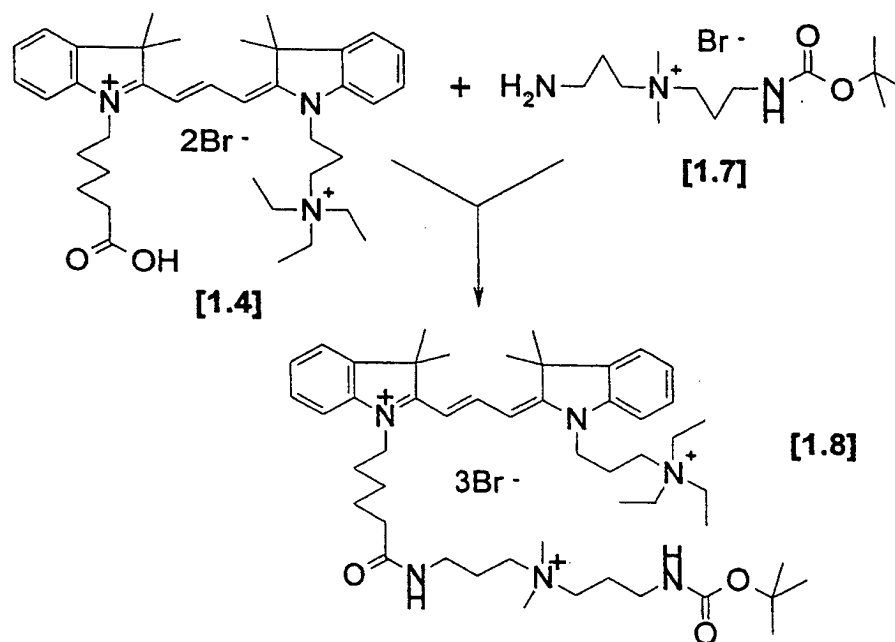
N-(3-aminopropyl)-N-((t-butoxycarbonylamino)propyl)-N,N-dimethylammonium bromide, [1.7]

N-(phthalimidopropyl)-N-((t-butoxycarbonylamino)propyl)-N,N-dimethylammonium bromide [1.6] (4.1g, 0.87mmol) was dissolved in ethanolic methylamine (33wt%, 8.02M, 10ml). The colourless solution was stirred at room temperature for 3 days, during which time a thick white precipitate formed (N,N'-dimethylphthalamide). The mixture was filtered; the solid was washed with a little cold ethanol. The filtrate was evaporated under reduced pressure to give an oil; this was triturated with ether and

dried under high vacuum to give the title compound [1.7] as a foam. Used without further purification.

δ_H (300MHz, CD_3OD) 1.43 (9H, s), 1.88-2.00 (4H, m), 2.75 (2H, t, J 6.8), 3.10 (6H, s), 3.15 (2H, t, J 6.6) and 3.3-3.5 (4H, m). A little N,N'-dimethylphthalamide also visible.

iii) Coupling of +2 charged Cy3 carboxy dye [1.4] to +1 charged linker [1.7]



10

Dye [1.4] (37mg, 50 μ mol) and O-(N-succinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (=TSTU, 17mg, 55 μ mol) were dissolved in dry acetonitrile (1ml). To the resulting deep pink-red solution was then added N,N-diisopropylethylamine (10 μ l, 57 μ mol) and the mixture stirred at room temperature with TLC monitoring (silica. Methanol, 50: water, 50. Saturated with NaBr. Free acid [1.4], R_f = 0.4 \rightarrow active ester, R_f = 0.5). Once the activation was complete (1hr), the amine [1.7] was added in portions, until TLC (as above) showed \approx complete conversion

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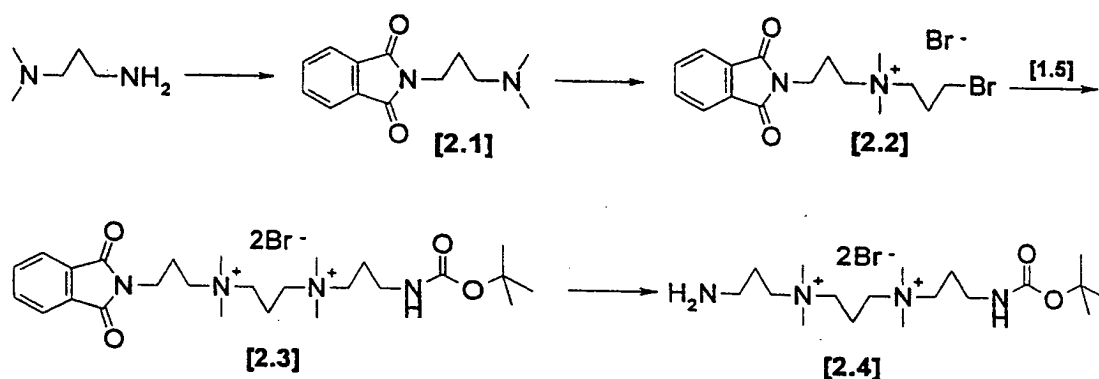
(active ester $R_f = 0.5 \rightarrow$ [1.8] $R_f = 0.35$). The solvent was then evaporated under reduced pressure; the residue was triturated with ether to give a brassy-coloured powder. Purified by preparative TLC, twice (silica, 20x20x0.1cm with concentration zone. Methanol, 50: water, 50. Saturated with NaBr). The main pink band was scraped off and extracted with the eluant, then methanol. The solvent was removed under reduced pressure and the residue dried. Product dye was extracted from the NaBr using chloroform; again the solvent was removed under reduced pressure, to give the title compound [1.8], 24mg.

10 UV/VIS λ_{\max} (MeOH); 548nm

δ_H (300MHz, CD_3OD) 1.34 (9H, t, J 7.2), 1.41 (9H, s), 1.58 (2H, m), 1.67-2.03 (20H, m. Includes 2xs for *gem*-dimethyl groups), 2.25-2.31 (4H, m), 3.09 (6H, s), 3.13 (2H, t, J 6.3), 3.26 (2H, t, J 6.3), 3.3-3.5 (10H, m, partially obscured by CHD_2OD), 3.60 (2H, app.t), 4.24 (2H, broad t, J 7.6), 4.33 (2H, broad t, J 7.4), 6.90 (1H, d, J 13.6), 6.92 (1H, d, J 13.2), 7.28-7.58 (8H, m) and 8.58 (1H, t, J 13.4).

15 Deprotection to the free amine is achieved using trifluoroacetic acid in methanol / chloroform (see example 2 for details of the method).

20

Example 2**+3 Charged dye, including +2 charged diamine linker (BOC-protected)****5 i) Preparation of a +2 charged diamine linker (BOC-protected)****N-(3-Dimethylamino)phthalimide, [2.1]**

10 3-Dimethylamino-1-propylamine (5.1g, 50mmol) and phthalic anhydride (8.15g, 55mmol) were mixed with chloroform (100ml); the resulting mixture was heated under reflux for 4.5hrs (CaCl₂ guard tube). After cooling the reaction mixture was washed twice with saturated aqueous sodium hydrogen carbonate solution, then with water. The organic solution was dried (MgSO₄), filtered and the solvent evaporated under reduced pressure to give a pale yellow oil. After drying under high vacuum, the title compound **[2.1]** was obtained, 10.2g (88%).

δ_H (300MHz, CDCl₃) 1.84 (2H, quin, *J* 7.2), 2.21 (6H, s), 2.34 (2H, t, *J* 7.3), 3.75 (2H, t, *J* 7.2), 7.70-7.74 (2H, m) and 7.82-7.86 (2H, m).

20

N-(3-Phthalimidopropyl)-N-(3-bromopropyl)-N,N-dimethylammonium bromide, [2.2]

N-(3-Dimethylamino)phthalimide **[2.1]** (2.32g, 10mmol) and 1,3-dibromopropane (4.04g, 20mmol) were dissolved in toluene (10ml) to

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give a clear solution. This was warmed to 80°C and stirred for 5hrs. A white precipitate formed. After cooling, this solid was collected, washed with toluene and ether, and dried under vacuum to give the product, 3.55g (82%).

5 δ_H (300MHz, D₂O) 2.08 (2H, m), 2.20 (2H, m), 2.97 (6H, s), 3.27-3.40 (6H, m), 3.63 (2H, t, *J* 6.6) and 7.66-7.73 (4H, m).

N-(3-Phthalimidopropyl)-N'-(3-(t-butoxycarbonylamino)propyl)-N,N,N',N'-tetramethyl-1,3-propanediammonium dibromide, [2.3]

10 N-(3-Phthalimidopropyl)-N-(3-bromopropyl)-N,N-dimethylammonium bromide [2.2] (4.34g, 10mmol) and N-(t-butoxycarbonyl)-N-(3-dimethylamino)propylamine [1.5] (2.02g, 10mmol) were mixed with acetonitrile (20ml) and set stirring. The mixture was heated to 60°C, but not all solid dissolved. More acetonitrile was added in
15 portions until an extra 20ml had been added, whereupon all solids dissolved. This solution was allowed to react at 60°C for 16hrs. After this time the solution was cooled and the solvent evaporated under reduced pressure; the resinous foam that resulted was triturated with ether, then dried under high vacuum to give the title compound as a powder, 6.4g
20 (100%).

δ_H (300MHz, CD₃CN) 0.86 (9H, s), 1.45 (2H, m, partly obscured by CHD₂CN), 1.66 (2H, m), 2.08 (2H, m), 2.58-2.70 (overlapping 6H, s + 6H, s + 2H, quartet), 2.86-3.06 (8H, m), 3.22 (2H, t, *J* 6.2), 5.70 (1H, broad app. t) and 7.23-7.32 (4H, m).

25

N-(3-Aminopropyl)-N'-(3-(t-butoxycarbonylamino)propyl)-N,N,N',N'-tetramethyl-1,3-propanediammonium dibromide, [2.4]

N-(3-Phthalimidopropyl)-N'-(3-(t-butoxycarbonylamino)propyl)-N,N,N',N'-tetramethyl-1,3-propanediammonium dibromide [2.3] (6.4g, 10mmol) was mixed with
30

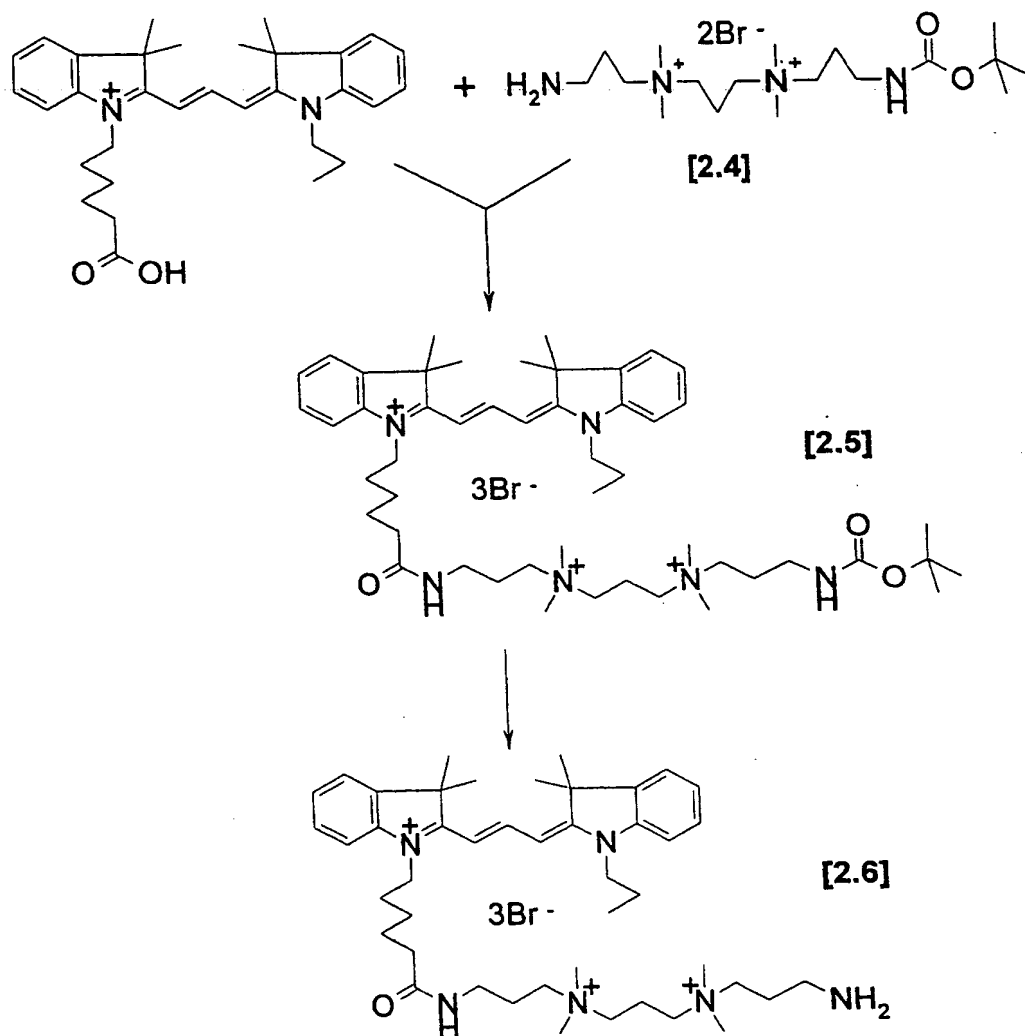
- 30 -

ethanolic methylamine (33wt%, 8.02M, 10ml) and set stirring. This was slow to all dissolve, so another 10ml of reagent added; after a while all the resinous mass had dissolved. The mixture was then left to stir for 3 days at room temperature. During this time a white solid precipitated (N,N'-
5 dimethylphthalamide). This was removed by filtration and then rinsed through with a little cold ethanol. The filtrate was evaporated under reduced pressure; the residue was redissolved in ethanol and re-evaporated, twice. The final residue was triturated with ether and dried under high vacuum to give the title compound as a white solid (extremely
10 deliquescent). This was stored under argon and used without further purification.

δ_H (300MHz, CD₃OD) 1.34 (9H, s), 1.82-1.93 (4H, m), 2.28-2.39 (2H, m), 2.68 (2H, t, *J* 6.6), 3.02-3.18 (14H, m) and 3.42 (8H, app. quin.). A little N,N'-dimethylphthalamide also evident.

15

ii) Coupling of +1 charged Cy3 carboxy dye to +2 charged linker [2.4]



- 5 1-Propyl-1'-(carboxypentyl)indocarbocyanine dye (25mg, $\approx 50 \mu\text{mol}$) was dissolved in dry acetonitrile (2ml), with stirring at room temperature. To the resulting pink-red solution was added O-(N-succinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (=TSTU, 18mg, $60 \mu\text{mol}$) and N,N-diisopropylethylamine (5 μl , $60 \mu\text{mol}$). Conversion
- 10 to the active ester was monitored by TLC (silica. Methanol, 20: chloroform, 80. Carboxy dye, $R_f = 0.3 \rightarrow$ active ester $R_f = 0.5$).

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After 1hr the amine [2.4] was added in portions, with TLC monitoring (silica. Methanol, 50: water, 50. Saturated with NaBr. Active ester $R_f = 0.55 \rightarrow$ [2.5] $R_f = 0.45$). Once reaction was deemed to be complete the solvent was removed under reduced pressure, the residue

5 trituated with ether and dried under high vacuum

The crude product was purified by prep. TLC (silica, 20x20x0.2cm with concentration zone. Methanol, 50: water, 50. Saturated with NaBr. Loaded in methanol solution). The main pink band was scraped off and extracted with the eluant, then methanol. The solvent was removed

10 under reduced pressure and the residue dried. Product dye was extracted from the NaBr using chloroform; again the solvent was removed under reduced pressure, to give the title compound [2.5], 30mg. The compound was not characterised further but subjected to an amine deprotection. UV/VIS λ_{max} (MeOH); 548nm.

15

Deprotection of amino group to give free amino dye

Compound [2.5] (30mg) was dissolved in 10% methanol / chloroform (2ml); trifluoroacetic acid (0.5ml) was then added and the mixture stirred at room temperature. Deprotection monitored by TLC (silica.

20 Methanol, 50: water, 50. Saturated with NaBr. [2.5] $R_f = 0.55 \rightarrow$ [2.6] $R_f = 0.7$). After 3hrs the reaction was halted and the solvent removed under reduced pressure. The residue was trituated with ether and dried under high vacuum.

Purified by prep.TLC (silica, 20x20x0.2cm with concentration

25 zone. Methanol, 50: water, 50. Saturated with NaBr. Loaded in methanol solution). The main pink band was scraped off and extracted with the eluant, then methanol. The solvent was removed under reduced pressure and the residue dried. Product dye was separated from NaBr using a flash plug of activated charcoal. The crude dye was loaded in water and the plug

30 eluted with water, methanol, then methanol, 1: chloroform, 1 to remove

dye. The solvent was evaporated and the residue dried under high vacuum to give the title compound [2.6], 10mg.

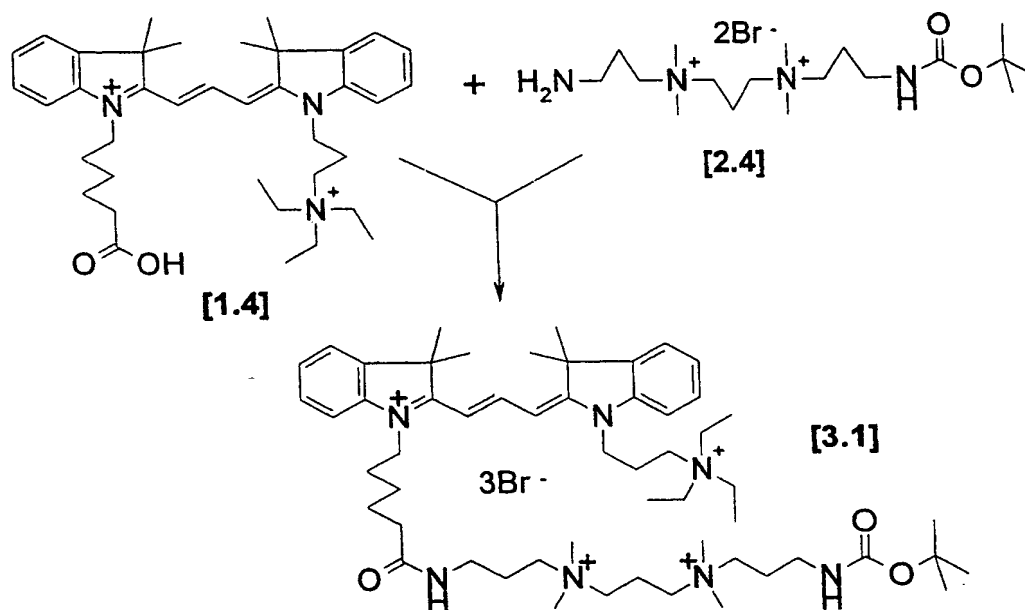
δ_H (300MHz, CD_3OD , broadened peaks) 1.08 (3H, t, J 7.3), 1.52 (2H, m), 1.6-1.9 (20H, m), 2.02 (2H, m), 2.14-2.39 (6H, m), 3.09 (2H, app. t), 3.18-3.3 (14H, m), 3.45-3.65 (8H, broad m), 4.11-4.19 (4H, m), 6.51 (1H, d, J 13.2), 6.53 (1H, d, J 13.6), 7.28-7.56 (8H, m) and 8.55 (1H, t, J 13.4).

UV/VIS λ_{max} (MeOH); 548nm.

10

Example 3

+4 Charged dye, including +2 charged diamine linker (BOC-protected)



15

The +2 charged carboxy dye [1.4] (37mg, $\approx 50\mu\text{mol}$) and O-(N-succinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TSTU, 17mg, $55\mu\text{mol}$) were dissolved in dry acetonitrile (1ml) to give a deep pink-red solution. To this was added N,N-diisopropylethylamine ($10\mu\text{l}$, $57\mu\text{mol}$);

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the resulting solution was left to stir at room temperature. The reaction was monitored by TLC (silica. Methanol, 50: water, 50. Saturated with NaBr.

[1.4] $R_f = 0.45 \rightarrow$ active ester, $R_f = 0.6$).

After 1hr the +2 amine linker, [2.4], was added portionwise
5 with further TLC monitoring (as above. NHS ester, $R_f = 0.6 \rightarrow$ [3.1]
 $R_f = 0.4$). Once the reaction appeared to be complete the solvent was
evaporated under reduced pressure; the residue was left to stand under
ether overnight. The ether was then decanted and the residue purified by
prep. TLC (silica, 20x20x0.1 cm. Methanol, 50: water, 50. Saturated with
10 NaBr. Loaded in methanol solution). The main pink band was scraped off
and extracted with the eluant, then methanol. The solvent was removed
under reduced pressure and the residue dried. Product dye was extracted
from the NaBr using chloroform; again the solvent was removed under
reduced pressure, to give the title compound [3.1], 40mg.

15 δ_H (300MHz, CD_3OD) 1.34 (9H, t, J 7.2), 1.42 (9H, s), 1.57
(2H, app. quin.), 1.70-2.01 (20H, m), 2.19-2.36 (6H, m), 3.13-3.20 (6H, s +
6H, s + 2H, partly obscured), 3.3 (2H, m, partly obscured by CHD_2OD),
3.33-3.64 (16H, m), 6.87 (1H, d, J 13.6), 6.90 (1H, m, J 13.2), 7.28-7.59
(8H, m) and 8.58 (1H, t, J 13.4).

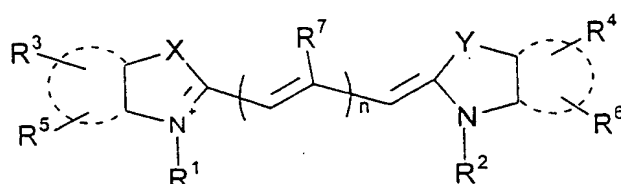
20 UV/VIS λ_{max} (MeOH); 548nm.

Deprotection to the free amine is achieved using
trifluoroacetic acid in methanol / chloroform (see example 2 for details of
the method).

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CLAIMS

- 5 1. A cyanine dye having the structure



(1)

where the dotted lines represent the carbon atoms necessary
for a one ring or a two or three fused ring system with 5 or 6 carbon atoms
10 in each ring and R^3 , R^4 , R^5 and R^6 attached to the rings,

X and Y are independently selected from O, S and CR_2^8 ,

where R^8 is $C_1 - C_4$ alkyl,

n is 1, 2 or 3,

15 at least one of R^1 , R^2 , R^3 , R^4 , R^5 , R^6 and R^7 optionally
comprises a reactive or a functional group,

at least one of R^1 , R^2 , R^3 , R^4 , R^5 , R^6 and R^7 incorporates one
to five positively charged nitrogen or phosphorus or sulphur atoms,

any remaining R^3 , R^4 , R^5 and R^6 is independently selected
from H, SO_3^- , Cl, Br, OR^9 and SR^9 , where R^9 is $C_1 - C_{10}$ alkyl or aryl or
20 aralkyl,

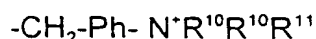
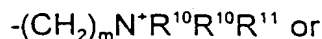
any remaining R^1 and R^2 is independently selected from
 $C_1 - C_{10}$ alkyl or aryl or aralkyl either unsubstituted or substituted by SO_3^- ,

any remaining R^7 is selected from H and $C_1 - C_{10}$ alkyl or aryl
or aralkyl either unsubstituted or substituted by SO_3^- ,

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provided that at least two positively charged atoms selected from nitrogen and phosphorus and sulphur are present in the groups R^1 , R^2 , R^3 , R^4 , R^5 , R^6 and R^7 .

2. A cyanine dye as claimed in claim 1, wherein the first atom of
5 R^7 (through which it is linked to the rest of the molecule) is H or C.
3. A cyanine dye as claimed in claim 1 or claim 2, having an overall positive charge of +2 to +6.
4. A cyanine dye as claimed in any one of claims 1 to 3, wherein a functional group, selected from primary amine, secondary amine,
10 hydrazine, hydroxylamine, pyrazolone, sulphydryl, carboxyl, hydroxyl, thiophosphate, imidazole and carbonyl including aldehyde and ketone, is present in at least one of R^1 , R^2 , R^3 , R^4 , R^5 , R^6 and R^7 .
5. A cyanine dye as claimed in any one of claims 1 to 3, wherein a reactive group, selected from succinimidyl ester, isothiocyanate,
15 dichlorotriazine, isocyanate, haloacetamide, maleimide, sulphonyl halide, acid halide, alkylimido ester, arylimido ester, carbodiimide, phosphoramidite, anhydride and acyl azide, is present in at least one of R^1 , R^2 , R^3 , R^4 , R^5 , R^6 and R^7 .
6. A cyanine dye as claimed in any one of claims 1 to 5, wherein
20 at least one of R^1 , R^2 , R^3 , R^4 , R^5 , R^6 and R^7 is a branched or straight chain of up to 60 carbon atoms or comprising amino acid residues and incorporates one to five positively charged nitrogen atoms.
7. A cyanine dye as claimed in claim 6, wherein a branched or straight chain of up to 60 carbon atoms incorporating one to five positively
25 charged nitrogen atoms has the structure



where m is 1 to 4,

R^{10} is $C_1 - C_{10}$ alkyl,

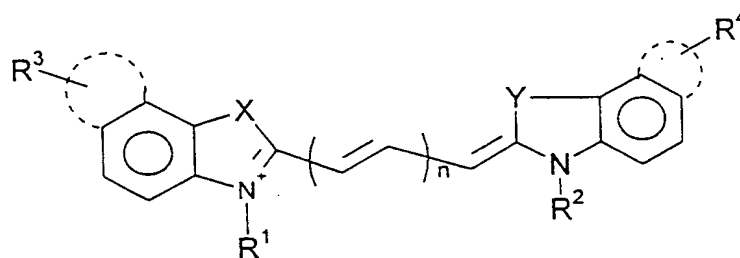
30 and R^{11} is $C_1 - C_{10}$ alkyl or $-(CH_2)_m N^+ R^{10} R^{10} R^{11}$.

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8. A cyanine dye as claimed in any one of claims 1 to 5, wherein at least one of R^1 , R^2 , R^3 , R^4 , R^5 , R^6 and R^7 has the structure $-L-Q$ where L is a linker and Q is the reactive or functional group.

9. A cyanine dye as claimed in any one of claims 1 to 6, wherein a linker is a straight chain of 1-60 atoms selected from C, N, O, S and P.

10. A cyanine dye as claimed in claim 1 and having the structure
(2)



(2)

wherein X and Y are $C(CH_3)_2$

n is 1 or 2,

R^1 is $-(CH_2)_5-COOH$,

R^2 is $-(CH_2)_3-N^+(C_2H_5)_3$ or

$-(CH_2)_3-N^+(CH_3)_2-(CH_2)_3-N^+(CH_3)_2(C_2H_5)$,

and R^3 and R^4 are H.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/02232

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C09B23/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C09B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 464 543 A (BASF AG) 8 January 1992 see claims 1-3; example 20 ---	1-10
A	DATABASE WPI Section Ch, Week 9729 Derwent Publications Ltd., London, GB; Class B02, AN 97-316532 XP002050337 & JP 09 124599 A (DOJIN KAGAKU KENKYUSHO KK), 13 May 1997 * compound F * see abstract --- -/--	1-10

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"&" document member of the same patent family

Date of the actual completion of the international search

16 November 1998

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INTERNATIONAL SEARCH REPORT

Int: onal Application No

PCT/GB 98/02232

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE 39 12 046 A (UNIV CARNEGIE MELLON) 15 March 1990 see claims; examples & US 5 268 486 A cited in the application & US 5 486 616 A cited in the application -----	1-10
A	WO 96 13552 A (MOLECULAR PROBES INC) 9 May 1996 see claims 1-21 -----	1-10
A	WO 96 00902 A (BIOMETRIC IMAGING INC ;LEE LINDA G (US); WOO SAM L (US)) 11 January 1996 see claims 1-22 -----	1-10
A	WO 97 17076 A (BIOMETRIC IMAGING INC) 15 May 1997 see claims 1-25; examples; tables -----	1-10
A	WO 96 33406 A (UNIV CARNEGIE MELLON) 24 October 1996 cited in the application see page 10, line 35 - page 14, line 4 -----	1-10

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/GB 98/02232

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0464543 A	08-01-1992	DE 4021078 A DE 59102718 D JP 4252268 A	09-01-1992 06-10-1994 08-09-1992
DE 3912046 A	15-03-1990	JP 2191674 A JP 2757965 B JP 10096727 A JP 10088012 A US 5486616 A US 5569766 A US 5569587 A US 5268486 A	27-07-1990 25-05-1998 14-04-1998 07-04-1998 23-01-1996 29-10-1996 29-10-1996 07-12-1993
WO 9613552 A	09-05-1996	US 5658751 A AU 3967295 A EP 0740689 A JP 9507879 T	19-08-1997 23-05-1996 06-11-1996 12-08-1997
WO 9600902 A	11-01-1996	US 5453505 A AU 3008595 A CA 2194150 A EP 0769145 A	26-09-1995 25-01-1996 11-01-1996 23-04-1997
WO 9717076 A	15-05-1997	US 5734058 A AU 1117797 A	31-03-1998 29-05-1997
WO 9633406 A	24-10-1996	AU 5557396 A CA 2218528 A EP 0821787 A	07-11-1996 24-10-1996 04-02-1998